

The Development of Cordyceps agariciformia (Bolt.) Seaver

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THE DEVELOPMENT OF CORDYCEPS AGARICIFORMIA¹

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(WITH PLATES 28 AND 29 AND 1 TEXT FIGURE)

INTRODUCTION

The members of the genus *Cordyceps* constitute a unique group of approximately two hundred species. With the exception of two species (*Cordyceps agariciformia* (Bolt.) Seaver and *C. parasitica* (Willd.) Seaver) which attack the hypogeous fruits of the fungus *Elaphomyces*, all these species occur as parasites on insects or spiders.

Our knowledge of the biology of *Cordyceps* is essentially limited to early studies of entomogenous species (de Bary, 4, 5; Tulasne, 40; Sopp, 39; Atkinson, 1; Pettit, 36). These investigators confined their studies to a single stage in the cycle, principally the development of the perithecium and ascospores (Fisch, 15; Lewton-Brain, 29; Maire, 30) because they lacked sufficient material to study the entire cycle of development or because they were handicapped by the lack of suitable microtechnical methods. Recently, Varitchak (41, cf. also Varitchak, 42) contributed to our knowledge of the morphological and cytological development of one of the entomogenous species. Facts established from studies on supposedly closely allied genera (Vincens, 45; Killian, 25; Gäumann, 17) have been employed as a means of arriving at an understanding of the genus *Cordyceps*.

In the present investigation an attempt is made to follow the course of development, both morphological and cytological, of *C. agariciformia* from the period extending from the sclerotial stage to the maturation of the ascospores. There has been to date only one such investigation (Varitchak, 41) and since this work was

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done on an altogether different species, the present contribution should afford opportunity for comparison and contrast.

MATERIALS AND METHODS

The material of *Cordyceps agariciformia* consisted of clavae ranging in stages of development from those which had emerged to a height of only a few millimeters above the parasitized *Elaphomyces* fruits up to and including clavae which bore mature perithecia and spores. The first collection of material was made at Gainesville, Florida, fixed in Gilsons fluid on February 24, 1924 and sent to the writer in November, 1932. The second collection included a rather complete series of developmental stages and was received in living condition from Cocoa, Florida on January 17, 1933. The third collection consisted of more mature stages and was sent from Gainesville on February 4, 1933. This living material, on arrival, was still attached to its host and was apparently healthy. The material was immediately washed in running water and fixed in formalin-acetic-alcohol, care being taken to cut the larger clavae into appropriate sizes to permit rapid penetration of the fixative. Due to the presence of adhering and incorporated sand particles on the *Elaphomyces* fruits and in the very young clavae, it was found necessary to desilicify these, after fixation, with hydrofluoric acid. The material was then dehydrated and embedded in paraffin. Sections were cut five, eight and ten microns in thickness in transverse, radial and tangential planes.

Various stains and combinations of stains were used, as Gram's Gentian violet (Couch, 10), Heidenhain's iron alum haematoxylin in combination with fast green, the same in combination with orange III in clove oil, safranin alone and Heidenhain's iron alum haematoxylin alone. While Gram's stain proved very useful in differentiating the structure of the opercula of the asci, walls of the asci and vegetative mycelium, and especially as a differential stain to show the relation of the mycelium to that of *Elaphomyces*, Heidenhain's iron alum haematoxylin used alone proved superior for the finer details of nuclear structure. The sections were in all cases cleared in clove oil, and in most cases this was followed by a treatment with cedar oil before passing them into xylol and mounting in balsam. The haematoxylin was applied in general

according to the schedule of Gwynne-Vaughan (Gwynne-Vaughan and Williamson, 20) though for some stages shorter periods of staining were found to be adequate.

HOST RELATIONS

DeBary (4, 5) has given a remarkable account of the phenomena attendant on infection, the formation and circulation of endogenously produced conidia in the "blood stream" and their germination to form sclerotia in the bodies of larvae of *Gastropacha Euphorbiae*, by the entomogenous species, *Cordyceps militaris*. Sopp (39) found that *C. norvegica* Sopp is normally a saprophyte on the forest floor, its parasitism on *Gastropacha Pini* being facultative. The results of his numerous inoculation experiments confirmed those of de Bary, e.g., that a given species of *Cordyceps* is not necessarily confined to a given species, genus or even group of insects. Lewton-Brain (29) reported the occurrence of the parasitic hyphae of *C. ophioglossoides* (Ehrh. Link (*C. parasitica* (Willd.) Seaver) in close contact with, and in some cases actually cemented to, the hyphae of *Elaphomyces*. In such cases a thin spot was apparent on the *Elaphomyces* hypha opposite the point of contact with the hypha of the parasite. Cells were likewise seen that had been penetrated by the parasite but no well-defined haustoria were seen. Lewton-Brain also reported the presence of isolated groups of cells of the host scattered about in the base of the clava of the parasite.

Within the base of the clava proper, in young clavae, the writer noted that the isolated groups of host cells were rather completely destroyed but in the surrounding areas the host cells were in various states of disorganization. In a few instances the hyphae of the parasite could be traced for considerable distances and in such cases a clearer picture of their activity could be formed. Although the point could not be conclusively proved with this material, there is considerable evidence that the *Cordyceps* mycelium is, in the early stages of its parasitism, an interhyphal parasite. A few favorable sections showed that narrow hyphae, much narrower than the parent hyphae, had penetrated the host cells (PLATE 29, FIG. 43). Whether these hyphae could be regarded as haustoria is, of course, open to debate. In such cases the cytoplasmic

contents of the host cells had not been completely destroyed. Other observations showed the mycelium of the parasite to be definitely intrahyphal, but in such cases the cytoplasmic contents of the host cells were almost or quite completely destroyed (PLATE 29, FIG. 45). Such a relation would be, if substantiated, entirely in keeping with the relation existing between many fungous parasites and their hosts.

Evidence indicates, likewise, that the clava of this species has its origin from numerous hyphae which arise as products of germination of certain cells of the pseudosclerotium situated just beneath the cortex of the *Elaphomyces* fruit body. This type of initiation has been described for *Claviceps purpurea* (Killian, 25) and *C. microcephala* (Vincens, 45). Also observations by the writer on the origin of the clavae of *Cordyceps clavulata* (Schw.) Ellis & Ev. show that in this species also the clava arises in the same manner as has been described above.² Should these observations prove to be correct in the case of *C. agariciformia*, Lewton-Brain's comparison of the base of the clava to the 'foot' of the *Anthoceros* or of the fern embryo, and his statement to the effect that the tissue of the host is destroyed and replaced by the "advancing 'foot' of the *Cordyceps*" are unwarranted. In the light, then, of evidence obtained from *Claviceps*, *Cordyceps clavulata*, various other entomogenous fungi (Petch, 35; Massee, 31; Cooke, 9; Vincens, 43) and indeed a majority of fungous parasites it is most probable that the parasite established its relation with the host long before clava formation began and has long since assimilated an abundance of food and stored it within the cells of its mycelium in preparation for its reproductive processes.

THE FORMATION AND STRUCTURE OF THE CLAVA

The earliest stages studied consisted of young clavae, a few millimeters long, which were sectioned longitudinally. Such sections showed very clearly that the emerging hyphae had ruptured the cortex of the host and in so doing had left groups of broken host cells scattered among the hyphae of the clava. Just how this rupture was accomplished is not known, but the evidence available indicates that the mechanics of the process are similar to that

² Paper to be published at a later date.

found in *C. clavulata*. In the latter, the hyphae arising from the germination of the sclerotial cells just beneath the integument of the host apply their tips against the cuticular covering and work their way through by dissolving a passageway or by mechanical pressure or by a combination of both processes. In other cases, individual hyphae emerge through natural openings in the cuticle. Though the hyphae emerge singly at first, they always emerge in definite groups so that the cuticular covering of the insect is riddled like a sieve at various points over its surface. Ultimately the continued pressure from the growth of other hyphae pushing up through these groups ruptures the cuticle at various points and the clavae emerge as cushion shaped structures, quite compactly organized toward the center, but of a loose, even floccose organization along the periphery. Bits of the cuticle which remain lodged among the hyphae of the clavae remain in the same relative position due to the predominant apical growth of the hyphae, so that later these fragments of cuticle are seen scattered among the hyphae at the base of the clavae.

In the young stages of *C. agariciformia*, the clava was composed of slightly larger and more compacted hyphae along the central axis. In sections of somewhat older specimens, ten to eleven millimeters long, this central region was composed of distinctly larger, longitudinally coursing hyphae intertwined with other very narrow ones. At the periphery, especially near the apex, the central region had given rise to a thin external layer of exceedingly fine and intricately interwoven hyphae which formed the outermost covering of the clava. In longitudinal sections of this stage, even prior to staining, the hyphae of the central region seem to form the skeleton that gives rigidity to the clava. Otherwise there was no differentiation of parts either as regards color or structure in the clavate, opaque head. This early differentiation was not noted in *C. militaris* (Varitchak, 41).

It was at first thought that the fertile hyphae which ultimately initiate the perithecia might possibly be differentiated in the very young clava, even before emergence from the *Elaphomyces* fruit body. Accordingly, the younger stages of clava formation were all cut in median longitudinal sections, in the hope of finding fertile hyphae differentiated in the pseudosclerotium. Careful examina-

tion of all sections of this stage of development failed to give any evidence of such differentiation, nor could the hyphae from which the fertile hyphae arose during later stages of clava formation be traced for any considerable distance due to the interweaving of the hyphae with each other. These longitudinally cut sections were especially fortunate in another respect, however, for they showed the relative position, the relative time of origin and the relative rapidity of development of perithecia all in one section. Sections of clavae ranging from nine to twelve millimeters in length and which had become differentiated into a whitish-yellow stipe of about eight-tenths millimeter in diameter by two millimeters in length showed very well the above mentioned internal features. By the time the clava has reached this stage of external differentiation, the interior parts have likewise become highly differentiated. The structure of the stipe is somewhat more homogeneous than during the early stages of development due to the fact that the hyphae have become more uniform in size. One can, however, still distinguish hyphae which are broad in diameter intermixed with and partially obscured by hyphae which are smaller in diameter. The periphery of the stipe is covered by a thin layer of loosely interwoven, hyaline hyphae. By far the greatest amount of differentiation is evident in the head. The central portion of the head is composed of the same hyphal types found in the stipe, while the periphery of the head is covered by a dense layer of pigmented, rather closely interwoven hyphae of the same size as the finer ones of the stipe. This layer is very thin near the base of the head where it gradually merges into the peripheral layer of the stipe. It is thicker near the equatorial region and tends to remain of the same thickness on upward over the apex of the head. Between this peripheral layer and the central core of the head an intermediate zone of hyphae is readily distinguishable because of its looser, web-like structure. All the hyphae composing this zone are at first of the same size as those of the peripheral layer but are hyaline and more loosely woven. The internal structure of the head of this species is therefore in agreement with that of *C. militaris* (Varitchak, 41) except that no interzonal layer was distinguished in the latter species. A study of younger stages shows that both the pigmented peripheral layer

and the colorless, loosely woven interzonal layer between this and the core had their origin from hyphae of the core. The hyphae composing these three well-defined zones are septate and the cells are, for the most part, uninucleate.

THE ORIGIN AND DEVELOPMENT OF THE ASCOGONIUM AND RELATED STRUCTURES

The ascogonia originate as branches from various hyphae of the interzonal region. In a few instances these structures were found to have originated from hyphae of the peripheral layer and in a like number of cases there was evidence of their origin from hyphae within the periphery of the core. Ascogonia of *C. militaris* originate in the peripheral layer (Varitchak, 41). The ascogonia, when first distinguishable, consist of short, usually slightly coiled, three to five septate hyphae, of uninucleate or binucleate cells. Unlike the aseptate ascogonia of *C. militaris* whose nucleus encloses a prominent nucleolus, these of *C. agariciformia* are distinguishable from the vegetative hyphae in their stages only by the fact that they are shorter, somewhat thicker, their nuclei stain more densely and they retain the stain more tenaciously than do the vegetative hyphae (PLATE 28, FIG. 1). In some instances, these structures arise in the larger interhyphal spaces of the interzonal region, and usually occur in groups of three to four. They were best seen in longitudinal sections of young clavae nine to twelve millimeters long. A careful study of the young heads throughout their entire length indicated that the first ascogonia arise near the equatorial region. A majority of the ascogonia found were below the equatorial region of the head, and a more or less complete series of developmental stages was to be noted as one examined the sections from the base up to the equatorial region. Thus, while ascogonia were being initiated in the basal portion of the head, sections through the equatorial region, and less markedly those in the apical region of the head, showed ascogenous hyphae beginning to appear in young perithecia. Development at the apex of the head is advanced beyond that at the base and that in the equatorial region is more advanced than either. These differences in degree of development of these regions were of great value in fixing the sequence of stages of development of

perithecia from the ascogonial stage up to the production of young ascogenous hyphae.

As the ascogonial cells increase in size they likewise tend to become more coiled and their cells become multinucleate. The nuclei, during these early stages of ascogonial development, were so small that positive identification of mitotic figures was not possible, yet the position of the nuclei seemed at times to indicate a recent division (PLATE 28, FIG. 2, 3). No antheridium-like structure was found in the vicinity of the ascogonia. Though two ascogonia often lay very close together, there was no evidence of union between them. This observation is in accord with the situation in *C. militaris* (Varitchak, 41). The possible presence of pores through the cross septa of the individual ascogonia through which nuclear exchange might occur, structures of rather frequent occurrence among apogamous ascomycetes, will be discussed later.

Concurrent with the development described above certain fine, deeply staining hyphae, much resembling vegetative hyphae, become conspicuous by coiling about and interweaving among the ascogonia (PLATE 28, FIG. 4, 5). These complexes are the "pelotons" of Varitchak. It seems entirely possible that some of these interweaving and coiling hyphae originate from the same hyphae which earlier gave rise to ascogonia. It could be quite easily demonstrated that many of these coiling hyphae originated as branches from the surrounding vegetative hyphae. During the early stages of this process the enveloping hyphae form no particular pattern; very soon, however, these smaller hyphae have increased in number to form a small spherical envelope with the ascogonia at the base (PLATE 28, FIG. 5). Still later the young perithecial envelope consists of many loosely compacted hyphal layers. During this development of the wall of the perithecium certain cells of the ascogonia enlarge considerably and their nuclei increase in number. According to Varitchak (41) the ascogonia of *C. militaris* during comparable stages are aseptate, multinucleate structures, the bases of which become inflated to form sacs that give rise to the ascogenous hyphae. It is evident from this study of *C. agariciformia* that the ascogonia are septate structures and that not all the cells give rise to ascogenous hyphae.

Another point of difference between the early stages of perithecial formation in *C. militaris* and *C. agariciformia* is in the manner in which the interweaving vegetative hyphae separate the various enlarged cells of two or more ascogonia which are developing close together. This seemed to indicate that two or more perithecia might arise from a single group of ascogonia. This was found, however, to be the case only very rarely. As a rule, these separating partitions of vegetative were later pushed aside by the developing ascogonia so that only one perithecial cavity resulted. In a few cases the cavities of two perithecia were observed to be confluent during the ascogenous hyphae stage; still more rarely the cavities of mature perithecia were only incompletely separated

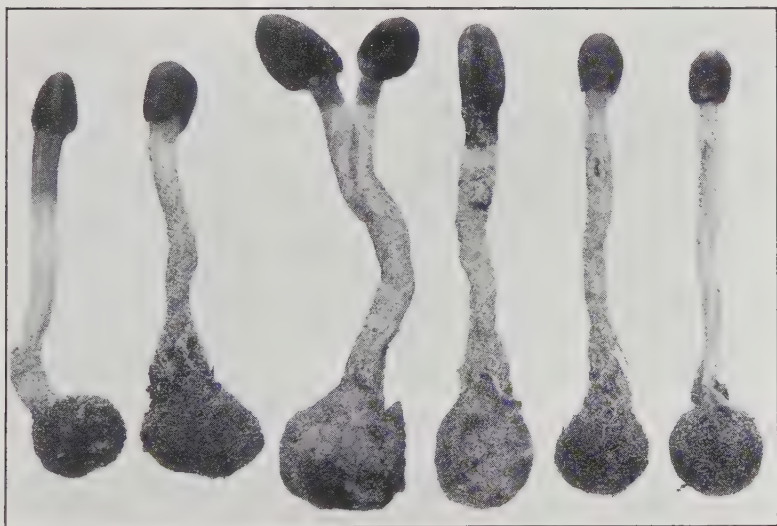


Fig. 1. Photograph of mature clavae attached to the host.

one from the other. These may either represent cases in which the separating hyphal partitions were not pushed aside until after they had been supplemented by others to form a distinct wall, or cases in which two perithecial primordia arose in close proximity to each other and in which, after their walls came in contact, these were displaced in part so that the cavities became partially confluent. Since the perithecia arise from a group of ascogonia, they may possibly prove to be always compound in origin.

DEVELOPMENT OF THE PERITHECIUM

One of the more striking features in the early stages of the perithecium is the development of its wall. Concurrent with the branching of the large cells of the ascogonia to form the primary ascogenous hyphae, to be discussed later, the spherical envelopes of loosely organized hyphal layers surrounding the ascogonia assume a definite polarity (PLATE 28, FIG. 6, 7). As the hyphae of this envelope increase in number the perithecial boundary opposite the ascogonia begins to elongate radially, i.e., toward the periphery of the head. For the sake of clarity in future discussion, this elongating side of the perithecium may be designated the ostiolar end, since the ostiolum will ultimately open there. By continued multiplication and growth of the hyphae of the walls, some of which may have originated from the ascogonia (Varitchak, 41) and grown up to form the inner wall layer of the perithecium, a tension is exerted on the hyphae of the ostiolar end. The hyphae of this region whose tips had heretofore overlapped are progressively pulled apart (PLATE 28, FIG. 9, 12). Elongation of some hyphae keeps pace with the separation of others so that during the stages in question the developing ostiolum is always closed at its distal end. The hyphae which are pulled apart, together with other short hyphae which arose by the branching of those making up the innermost layer of the wall, fall back into the developing cavity. In such a stage the perithecial cavity is rather completely lined by hyphae with free tips.

Concurrent with the appearance of polarity in the perithecium, the large, multinucleate cells of the ascogonia begin to branch variously (PLATE 28, FIG. 6, 7), nuclei migrate fortuitously out into the branches and when these become septate, more than two nuclei are often found in some of the cells. These branches, the primary ascogenous hyphae, grow into the developing perithecial cavity. At this stage hyphae which have their origin in the wall layers are seen to have grown among the primary ascogenous hyphae and ultimately to have formed a partition of vegetative hyphae between these and their bases in the ascogonia (PLATE 28, FIG. 7). Finally, the old, more or less completely emptied ascogonial cells are almost completely hidden by the dense growth

of hyphae between and about them. This is contrary to the situation in *C. militaris* where the ascogonia tend to disappear very early (Varitchak, 41). In other cases, perhaps just as typical, the ascogonial remains lie at the base, just beneath the floor of the perithecial cavity. At the level of the floor of the perithecial cavity some of the primary ascogenous hyphae multiply by branching and spread out along the floor of the perithecium, ultimately forming a layer of plectenchyma whose cells contain two to four nuclei (PLATE 28, FIG. 9, 13). These cells give rise to the true ascogenous hyphae, but due to their compactness and the presence of other hyphae it is impossible to state how many times a given ascogenous hypha branches before it gives rise to asci.

It is in sections of heads of about this stage that the cells of certain ascogonia are seen to be almost or quite emptied of protoplasmic contents. In certain favorably oriented sections of ascogonial cells, ruptures were noted through septa separating certain of these cells (PLATE 28, FIG. 10). An interpretation of these ruptures is difficult. The evidence in hand at present does not suggest any such sexual relation as has been described for *Polystigma rubrum* (Nienburg, 34), *Epichloe typhina* (Vincens, 45), *E. Bambusae* (Gaumann, 17), *Ophiobolus graminis* (Jones, 24), *Rhizina undulata* (Fitzpatrick, 16), or *Ascobolus citrinus* (Schweizer, 37). These ruptures were not observed in the younger ascogonia though they were sought for diligently. Their occurrence is due, in all probability, to mechanical injury by the closely investing hyphae, or else is the result of some tension caused by the growth of the parts about them. Even though no actual mitotic figures were seen, the multinucleate cells of the young ascogonia apparently result from ordinary mitotic divisions of the one or two nuclei originally present in each. Hence all the nuclei of a given ascogonial cell are believed to be daughter nuclei of the original nucleus or nuclei, rather than derived by the division of nuclei which have migrated from other cells of the ascogonium. This interpretation is in accord with that published for *C. militaris* (Varitchak, 41).

Even while the perithecia are in this early stage of development the hyphae in the interzonal region show the effects of crowding and stretching. As the perithecia continue their development,

these interzonal hyphae became progressively more disorganized, until finally at maturity the entire layer of perithecia together with the peripheral zone may be peeled away from the central core quite readily.

DEVELOPMENT AND CYTOLOGY OF THE ASCUS

This critical phase in the development of the fungus merits especial consideration since it has received more attention by earlier investigators than have the phases thus far discussed. As has been stated earlier, the asci grow out from ascogenous hyphae which have in turn arisen from the plectenchymatous floor of the perithecial cavity. In some cases the asci arise from typical croziers (PLATE 28, FIG. 14), but intermediate conditions have been noted between these and cases where the asci arise as direct outgrowths of binucleate cells of the ascogenous hyphae (PLATE 28, FIG. 13). When this latter type of origin is frequent along a given ascogenous hypha the picture is like that figured; but probably wrongly interpreted, for *Epichloe typhina* (Vincens, 45). Proliferation of croziers, as reported for *C. militaris*, were not observed nor were the ascogenous hyphae found to lose their contents and become amorphous as the asci matured (Varitchak, 41). In all cases the ascogenous hyphae and young asci are extremely small, which, combined with the fact that these structures are compactly arranged in the perithecium, makes it difficult to identify all the variations present.

Regardless of how it originates, the young ascus is always binucleate (PLATE 28, FIG. 15). These two nuclei are indistinguishable. Both are very small and stain homogeneously. None were seen which, prior to fusion, showed a hyalosphere nor could a centrosome be satisfactorily demonstrated such as was described in *C. militaris* (Varitchak, 41). These two nuclei approach each other and fuse before the young ascus has enlarged to twice its original length. The resulting primary ascus nucleus undergoes a considerable number of changes prior to its first division, all the details of which have not been heretofore published for any species of *Cordyceps*.

One of the more significant of these changes in the primary ascus nucleus is its enormous increase in size (PLATE 28, FIG.

16-26). Contrary to the situation in *C. militaris* (Varitchak, 41), the nucleus loses its homogeneity very early in its development and one can then readily distinguish the nuclear membrane, nuclear sap, reticulum and a relatively very large nucleolus. Certain sections show a densely staining granule in contact with the periphery of the nuclear membrane (PLATE 28, FIG. 19). Similar and like staining granules have been called centrosomes (Bagschee, 3; Varitchak, 41). It could not be definitely ascertained whether this granule is extranuclear or intranuclear in origin. During the early stages of nuclear enlargement the immense nucleolus is encased by the reticulum, though it appears in all cases to be situated near the periphery of the nucleus (PLATE 28, FIG. 16). Later, as the nucleus prepares itself for division, the reticulum becomes organized into a definite spireme. As this structure becomes more thread-like it tends to pull itself away from the nucleolus to the opposite side of the nucleus and is connected with the nucleolus by only a thread or two. At about this stage the nuclear membrane becomes indistinct. During the development of the spireme the nucleolus is somewhat smaller in size, and at the same time very small, densely staining, chromatin-like granules can be observed in the equatorial region of the nucleus (PLATE 28, FIG. 25). Some of the spireme thread are distinct even during such a stage as this. Although the fate of the nucleolus could not be followed so completely as it was in *Pustularia bolarioides* Ramsb. by Bagschee (3), the evidence in hand indicates that this body undergoes fragmentation just prior to and during the early stages of the first division of the nucleus. During the period of its growth and reorganization prior to its first division the nucleus is located centrally in the ascus and occupies practically the entire width of it.

The stages of nuclear division seen most clearly during this study were metaphases or anaphases; only rarely was a telophase or intermediate phases evident (PLATE 29, FIG. 27a, 27, 29, 31). Compared with those of subsequent divisions the spindle of the primary division is quite long (PLATE 29, FIG. 27a). Though little claim to certainty is made, because of their small size, four chromosomes were counted in preparations which showed particularly good metaphase and early anaphase conditions (PLATE 29, FIG.

27a). It seems from this and the counts which will be given later for subsequent divisions that the somatic chromosome number for *C. agariciformia* is two. Varitchak (41) states that two chromosomes were counted during the first division of the fusion nucleus, and later states definitely that the somatic chromosome number for *C. militaris* is two. During this first division, as likewise for the two succeeding divisions, a centrosome is apparent at each pole and is discoid to cap-shaped in form. The concave side of the centrosome is constantly adjacent to the spindle. Such a situation has been described by Jones (24) for *Ophiobolus graminis* Sacc. The elongated spindle is situated about centrally in the ascus and describes a wide arc, though it is not uncommonly an elongated S-shaped structure.

The two nuclei which reorganize from this division have a structure similar to the parent nucleus, differing from it principally in their smaller size and the absence of the large nucleolus; this structure being represented by two small, deeply staining, granules (PLATE 29, FIG. 28). In this respect these observations differ from those of Varitchak (41) who figures the interphase nuclei of *C. militaris* as homogeneous structures which lack both a reticulum and centrosomes. Judging from their size, internal structure and the infrequency with which these nuclei are found the second division must follow the first immediately. During all the nuclear divisions, except possibly the third, the cytoplasm is rather uniformly dense, though conspicuous vacuoles are present near the apex of the ascus.

The second division in the ascus is, as in other ascomycetes, a simultaneous division of the first two daughter nuclei. The form of the spindle differs little from that of the first division except in its somewhat smaller size (PLATE 29, FIG 29). During the late metaphase of this division four small chromosomes are apparent near the equatorial plate, and other figures of the early anaphase show two such deeply staining granules in transit to each pole. Evidence of astral rays extending from the centrosomes was seen in a few preparations, only. The definite, deeply staining spindle in most cases appears as a single line, though a truer picture of the spindle was sometimes seen. The spindle is so narrow that it is very difficult to demonstrate its individual

fibers (PLATE 29, FIG. 29). In fact, such fine cytological details are seen only when the differentiation of the stain is halted just at the right point during the process of destaining. This difficulty appears to be the rule rather than the exception in work with nuclei so small (Jones, 23, 24; Varitchak, 41). The present writer was able to solve this difficulty in part, and at the same time insure entire asci for the study of later cytological phenomena, by making use of smears of asci which were stained and studied in toto.

The four nuclei which reorganize from this second division are similar to the parent nuclei except for their smaller size (PLATE 29, FIG. 30). Although there appears to be a longer interval between the second and third divisions than between the first and second, the nuclei do not increase in size appreciably nor reorganize as resting nuclei. The third division is likewise simultaneous and the spindles are predominantly parallel to or but slightly oblique to the long axis of the ascus (PLATE 29, FIG. 31, 32). A similar orientation of these spindles was found in *C. militaris* (Varitchak, 41). Such an orientation of spindles results, of course, in a linear arrangement of the reorganizing nuclei. This arrangement seems particularly significant in connection with the form of the spores later to be organized about these eight nuclei.

The matter of how the filamentous, multiseptate, ascospores so characteristic of this genus are developed from the product of the third successive division of the fusion nucleus in the ascus has been the subject of diverse opinions (Lewton-Brain 29; Faull, 14; Maire, 30; Varitchak, 41). As Faull (14) has remarked, Lewton-Brain attempted a cytological investigation on *Cordyceps ophioglossoides* with material that was not properly fixed. In consequence he probably mistook nucleoli for nuclei. Although he figures no nuclear divisions, Lewton-Brain states that there is no evidence of spore delimitation in the ascus until after a great number of nuclei are present in it. Later, he says, these nuclei arrange themselves into eight rows and the cytoplasm about each row becomes delimited by longitudinal cleavages to form eight, multinucleate, aseptate spores; each of the spores becoming septate only after additional nuclear divisions. Faull (14) saw uni-

nucleate spores of the same species and thinks that the multinucleate spore probably arises from the uninucleate one by nuclear divisions accompanied by septation. Maire (30) found uninucleate spores in *C. agariciformia* but did not follow their development. Varitchak (41) in his work on *C. militaris* is in agreement with Faull.

The present writer finds that following the third division the cytoplasm about the eight reorganized nuclei cleaves to form an ascospore initial. No actual cleavage planes are noticeable until after the eight ascospore initials have completely reorganized, but even during the course of the third division the cytoplasm becomes particularly dense near the poles of the spindles. This condensation of cytoplasm continues until one can readily distinguish the symmetry of the young ascospore initials, even before cleavage planes are evident. A very careful study of the process of differentiation of the ascospore initials has failed to demonstrate that astral rays in any way initiate the cleavage of the cytoplasm during these stages. The spore initials seem rather to be first delimited by condensation of the cytoplasm about the nuclei followed by a series of small vacuoles which form and coalesce along the periphery of the spore initials, thus cleaving the cytoplasm. Later, after cytoplasmic cleavage has progressed considerably, astral-like rays can be demonstrated, in some preparations, emanating from a densely stained area at one end of the spore initial (PLATE 29, FIG. 37).

Thus from the evidence available, the mode of formation of the ascospore initials seems to accord rather with that described by Faull (14) and Jones (23, 24) than with that described by Harper (22) and Varitchak (41). It should be noted, however, that the ascospore initials in *Cordyceps militaris* are delimited by astral rays, according to Varitchak.

The ascospore initials when differentiated, lie somewhat obliquely to the long axis of the ascus and tend to occupy the entire width of the ascus (PLATE 29, FIG. 34-36). Judging from the staining reactions of the boundaries of these structures as compared with the surrounding epiplasm—and later, as compared with the staining reactions of the boundaries of somewhat older stages

of these structures—no spore wall is formed about the spore initial, merely a cytoplasmic membrane (PLATE 29, FIG. 34).

As in *C. militaris* (Varitchak, 41) the growth of these unicellular spore initials must take place extremely rapidly, for very few stages in their growth were found. In practically all stages seen, even the youngest, the nuclei were actively elongating indicating that the uninucleate spore initial stage is of short duration (PLATE 29, FIG. 34-39). In those stages found there was no appearance of cross septa until after the spore initial had grown considerably in length. Such a condition was described for *C. militaris*, but the suggestion was made that possibly the newly formed septa were difficult to stain. This explanation might suffice were it not that later cross septa are clearly evident, although the spores are elongating and nuclear divisions are still in progress. During the early growth of the spores the nuclei elongate greatly in such a manner as was described for *Rhytisma acerinum* (Jones, 23). Just how they become organized again is not clear (PLATE 29, FIG. 39). Later the nuclei divide mitotically but the figures are too small to show any detail except at anaphase (PLATE 29, FIG. 40). At this stage the small deeply stained spindles, with a mass of chromatin at each pole, are quite conspicuous. Even after transverse septa become apparent the spores continue to grow in length, each cell of the spore apparently being capable of independent elongation and division, though the terminal cells seemingly become inactive before the median ones do. The young spores are always eight in number and at maturity are thirty-five to fifty septate; each cell being uninucleate. Prior to being released from the ascus many spores break up into unicellular segments.

PECULIARITIES OF THE ASCUS

The development of the capitulum of the ascus first becomes noticeable as the primary ascus nucleus is preparing for its first division (PLATE 28, FIG. 19, 20). Such a stage shows the apex of the ascus somewhat enlarged and practically void of the granular contents so characteristic of the remainder of the ascus. Further differentiation proceeds rapidly so that by the time the three successive divisions of the nucleus have been accomplished the apical

portion of the ascus wall has become considerably thickened and a pore or core of clear cytoplasm is seen to extend up into the thickened area of the wall (PLATE 29, FIG. 33). At maturity, the apex of the ascus is covered by a much thickened, conical, lid-like process which is penetrated centrally by the narrow pore or ascostome. During the period in which the three successive primary nuclear divisions are accomplished the ascus has been increasing steadily in length, so that by the time the capitulum is completely formed the ascus has about reached its definitive length.

Another peculiar feature of the ascus, aside from certain deformities which perhaps never reach maturity or else become normal before they reach maturity, is due to the presence of crystals in it. These structures are quite evident in fresh material, and their effect is quite noticeable in sections prior to being stained. Apparently the various treatments through which the material was passed (fixation, dehydration, etc.) dissolved the crystals, but the distortion of walls and cytoplasm due to their presence were preserved (PLATE 29, FIG. 42). These crystals are noticeable, even in young asci, but as the asci approach maturity the distortion caused by the structures is quite pronounced. Often the ascus walls and even the spores are bulged to such an extent that rupture seems imminent. It is suggested that these crystals may play a rôle in liberation of the ascospores.

THE MATURE CLAVA AND PERITHECIUM

During the course of ascospore development, as described above, the clava has been steadily increasing in size. The length of the stipe seems to depend on the depth of the parasitized host under the soil, while the dimensions of the head are probably related to nutritional factors. The stipe is usually white to grayish-white in color while the head is greenish-black when the clava is mature (TEXT FIG. 1). Such mature heads, when placed under humid conditions, soon become covered by a hyaline, viscous mass of spore segments and almost complete spores. These spore segments swell considerably and often germinate while still in contact with the clava. On nutrient medium these germinating spore segments form abundant conidia resembling the form genus *Spicaria*.

The mature perithecium is ovate to pear-shaped in form, its broad base resting in the much weakened and distorted interzonal area and its long neck protruding through the pigmented peripheral layer to the outside. So evenly do the hyphae of the neck fit into those of the peripheral layer that, as suggested by Lewton-Brain (29), the perithecia appear on superficial examination with low magnifications as invaginations of the peripheral layer. At maturity the perithecial cavity is completely filled with asci in all stages of development. The paraphyses-like hyphae together with the hyphae which were dislocated during ostiolar development have gelatinized and disappeared for the most part, in all probability being used as food by the developing asci or else as an osmotic fluid during the enlargement of the perithecium. At the base of the perithecium may be found the asci which have emptied and collapsed, thus making room for a new crop of asci originating from the still active ascogenous hyphae. It is not known how long a perithecium continues to produce spores, but it is conceivable that the active ascogenous hyphae may continue to produce new asci and spores for weeks. The exterior walls of the mature perithecium, at its base, are very nearly in contact with those of the adjacent perithecia. The walls have, throughout, lost their plectenchymatous structure, being now pseudoparenchymatous. The ostiolum, which is now continuous and open from the perithecial cavity to the outside, is lined with deeply staining paraphyses which have arisen from the walls of the ostiolum. Scattered among the asci and often in the ostiolum (they were for the most part washed away) are many complete spores, but for the most part the spores have broken at the cross septa to form spore segments.

SUMMARY

1. This investigation consists of a study of the development of *Cordyceps agariciformia* (Bolt.) Seaver from the initiation of the clava until the spores mature.

2. This species begins its parasitism as an interhyphal parasite, but soon invades the hyphae of its host, *Elaphomyces*, and becomes an intracellular parasite.

3. Apparently the clava is formed after the parasite has com-

pleted the absorption of food from its host, and is initiated by hyphae which have arisen from the germination of pseudo-sclerotial cells lying just beneath the cortex of the host.

4. The young clava consists of a mass of hyphae, the central ones being somewhat larger in diameter and somewhat more compact than the peripheral ones. Very early, however, the stipe becomes differentiated externally into a stipe and head.

5. Internally, the stipe consists of (1) a central core of compact, hyaline hyphae of two sizes which run predominantly parallel to the long axis of the clava and (2) a peripheral layer of fine, hyaline, loosely interwoven hyphae. The head consists of (1) a central core, which structurally is a continuation of the core of the stipe, (2) a peripheral layer of fine, pigmented and intricately interwoven hyphae, and (3) an interzonal region of hyaline, loosely woven hyphae, of the same diameter as those of the peripheral layer.

6. The ascogonia arise predominantly within the interzonal region in groups of three to four as somewhat enlarged hyphae, each three to five celled. Certain cells of these structures rapidly enlarge and become multinucleate. No evidence of an antheridial structure was seen.

7. Fine, densely staining hyphae coil about and enclose the ascogonia and thus initiate the formation of perithecia.

8. The perithecial primordium is, during its earlier stages, more or less globular but soon the enveloping hyphae assume a polarity of growth and the true wall and ostiolum are formed.

9. Later the multinucleate cells of the ascogonium branch to form primary ascogenous hyphae. These hyphae elongate to form a plectenchymatous layer of binucleate cells along the floor of the perithecial cavity, which in turn give rise to the true ascogenous hyphae.

10. The ascus arises either from a crozier or as a direct outgrowth of a binucleate cell of an ascogenous hypha.

11. The cytology of the ascus and spores has been studied in considerable detail. The ascospore initials arise by vacuolar cleavage rather through the activity of astral rays. These structures are at first unicellular and uninucleate, but very soon elongate to form the mature, multiseptate spore. A septum is not formed

until after the spore initial has attained considerable length. Even after septa begin to form, the spores continue to increase in length by means of independent elongation and division of its cells, until at maturity the spore is thirty-five to fifty septate.

12. The somatic number of chromosomes for this species is two.

ACKNOWLEDGMENTS

In conclusion, I wish to express my deep feeling of gratitude to Doctor Duncan S. Johnson under whose direction this study was accomplished. His unfailing kindness and invaluable suggestions during the course of this study, and particularly in preparation of the original manuscript, have, in a very real sense, made the completion of this work possible.

I also wish to acknowledge my deep obligation to Doctor G. F. Weber of the University of Florida Agricultural Experiment Station, Gainesville, Florida for his kindness in sending me material of *Cordyceps agariciformia*; and to Doctor A. S. Rhoades of the University of Florida station at Cocoa, Florida for like material and also for an excellent photograph of these specimens. Likewise, I wish to acknowledge the kindness and suggestions of Doctor F. A. Wolf of Duke University during the course of this revision.

EXPERIMENT, GEORGIA.

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EXPLANATION OF PLATES

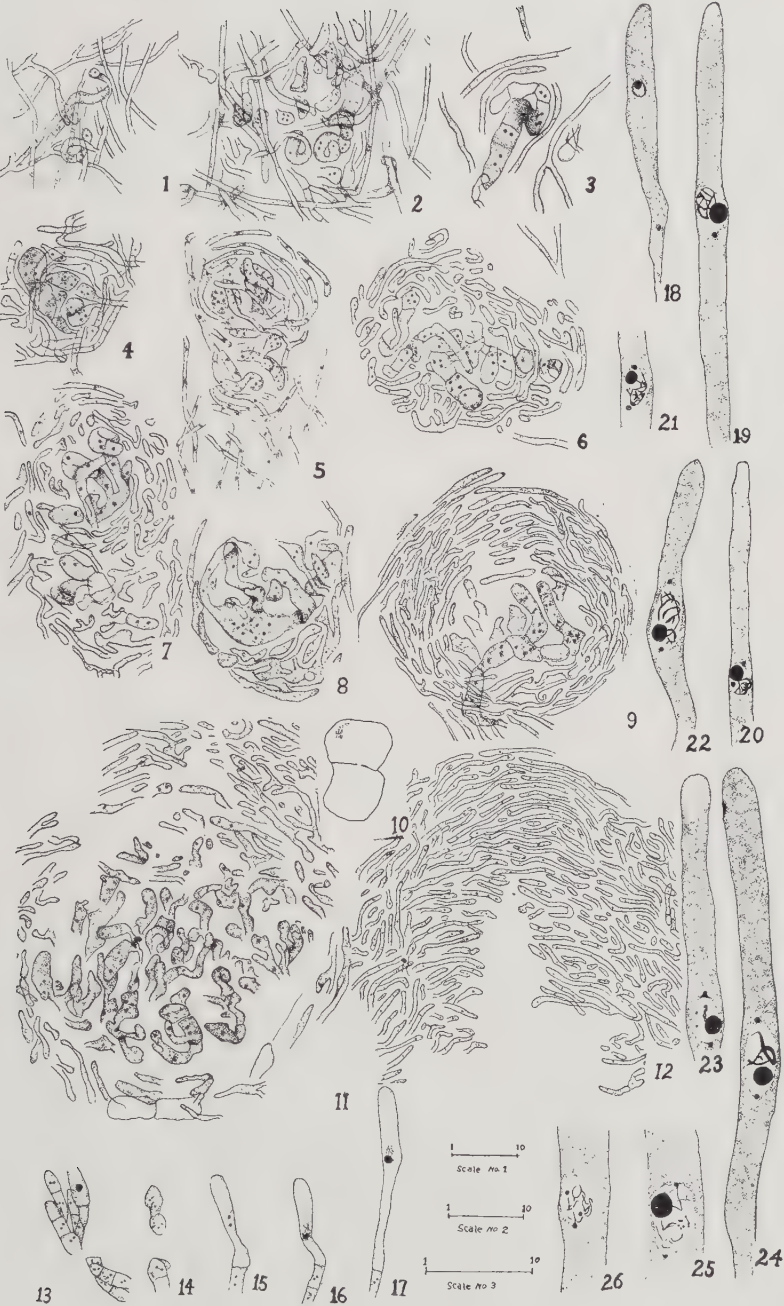
PLATE 28

All figures drawn with the aid of an Abbe camera lucida (Zeiss). Scale 1 Zeiss 3 mm. Apochr. immersion lens (N. A. I. 40) with Zeiss 20 × comp. ocular. Scale 2 Leitz $\frac{1}{12}$ immersion lens (N. A. I. 30) with Leitz 15 × periplan ocular. Fig. 1, 4, 5, 8, 10, 11, 12 drawn to Scale 1, others to Scale 2. Fig. 1, A group of young ascogonia which are slightly larger and stain more deeply than the surrounding vegetative hyphae; 2, Somewhat older ascogonia, showing a distinct coiling and enlargement; 3, A single ascogonium showing enlargement of certain of its cells; 4, Ascogonia in which certain cells have become multinucleate. Note enveloping hyphae; 5, Portions of ascogonia whose tips overlie each other, though there is no evidence of connections between them; 6, A group of ascogonia showing certain cells that are beginning to branch; 7, A more advanced stage than the preceding showing a few of the partition hyphae about the equatorial region; 8, Portion of an ascogonium showing manner of branching of much enlarged, multinucleate, ascogonial cells; 9, An obliquely cut section showing a few primary ascogenous hyphae in the young perithecial cavity; 10, Two, much enlarged, empty, ascogonial cells taken from the base of a young perithecium bearing ascogenous hyphae. A rupture in the septum is evident. 11, Semi-diagrammatic sketch of the interior of a young perithecium. The variety

in structure of the ascogenous hyphae is striking. 12, Apex of a young perithecium showing the mode of formation of perithecial wall and ostiolum. Fig. 13, 14, 15, 16, 17, 20, 21, drawn to Scale 1; Fig. 18, 23, drawn to Scale 2; others to Scale 3. Fig. 13, Portion of the plectenchyma of the floor of a young perithecium, showing asci arising directly from binucleate ascogenous hyphae; 14, Two views of croziers found at the base of a young perithecium; 15, A young, binucleate ascus; 16-26, A series showing the growth of the ascus and progress of nuclear changes prior to the first division. The polar granules present in each case are interpreted as centrosomes. Fig. 19, 20, 23, beginning of operculum formation.

PLATE 29

All figures drawn with the aid of an Abbe camera lucida (Zeiss). Scales as in Plate 28. Fig. 27a, 30, 34-45, drawn to Scale 1, others drawn to Scale 3. Fig. 27a and 27, First division of the primary ascus nucleus at early anaphase and metaphase respectively. 28, A binucleate ascus, the nuclei preparing for the second division; 29, Division figure of the second mitosis. Due to their small size, the behavior of the chromosomes at metaphase could not be ascertained. 30, A four nucleate ascus, the nuclei preparing for the third successive division; 31-32, Division of the third nuclear division, fig. 31 showing chromosomes, fig. 32 showing a late anaphase condition; 33, An eight nucleate ascus; 34-42, The organization of the spore initials and the growth of these to mature spores in order of sequence. Fig. 34, In preparations of this stage the activity of astral rays could not be detected. Fig. 35, The nucleus is shown elongating to the periphery of the spore initial. Fig. 37, Shows the crescent-shaped centrosome-like body at the basal pole of the elongating spore initial. Fig. 39, Elongated nuclei in young spores. Fig. 40-41, Nuclear division in cells of young spores. Fig. 42, Distortion of spores and ascus wall caused by crystals; Fig. 43-45, Illustrating certain phases of the host relations. Fig. 43, An intracellular hypha has pushed an attenuated tip into the adjoining host cell. Fig. 44-45, Intracellular hyphae of the parasite.



CORDYCEPS AGARICIFORMIA



CORDYCEPS AGARICIFORMIA

VITA :

Wilbert Armonde Jenkins was born at Pekin, North Carolina, September 16, 1905. His early training was obtained in the public schools of Durham, North Carolina. He entered Duke University, Durham, North Carolina, in 1924, and received the Bachelor of Arts degree in 1928, and the Master of Arts degree in 1929. During this period he held an undergraduate assistantship in Botany, 1926 - 1928, and a graduate assistantship in Botany, 1928-1929. The following two years, 1929-1931, were spent in graduate work in the department of Plant Pathology, Cornell University, Ithaca, New York. During this time he held an assistantship in Mycology. The Summers, 1929 - 1931, were spent as Instructor in Biology in the summer school affiliated with Duke University at Lake Junaluska, North Carolina, and as Instructor in Botany in the Duke University summer school at Durham, North Carolina. The academic year, 1931-1932, was spent as Instructor in Botany in Duke University.

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The title of his publication is: The Cherry leaf-spot fungus, *Mycosphaerella cerasella* Aderh., its morphology and life history. *Phytopath.* 20:329-337. 1930.

